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Title

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Permalink

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Journal

Endocrinology, 149(3)

ISSN

0013-7227

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Publication Date

2008-03-01

DOI

10.1210/en.2007-0927

Peer reviewed

Synergistic Impairment of Glucose Homeostasis in *ob/ob* Mice Lacking Functional Serotonin 2C Receptors

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To investigate how serotonin and leptin interact in the regulation of energy balance and glucose homeostasis, we generated a genetic mouse model, the OB2C mouse, which lacks functional serotonin 2C receptors and the adipocyte hormone leptin. The OB2C mice exhibited a dramatic diabetes phenotype, evidenced by a synergistic increase in serum glucose levels and water intake. The severity of the animals' diabetes phenotype would not have been predicted from the phenotypic characterization of mice bearing mutations of either the leptin (OB mutant mice) or the serotonin 2C receptor gene (2C

mutant mice). The synergistic impairment in glucose homeostasis developed at an age when OB2C mice did not differ in body weight from OB mice, suggesting that this impairment was not an indirect consequence of increased adiposity. We also demonstrated that the improvement in glucose tolerance in wild-type mice treated with the serotonin releaser and reuptake inhibitor fenfluramine was blunted in 2C mutant mice. These pharmacological and genetic findings provide evidence that the serotonin 2C receptor has direct effects on glucose homeostasis. (*Endocrinology* 149: 955–961, 2008)

SUSCEPTIBILITY TO OBESITY and type 2 diabetes is known to be associated with a polygenic mode of inheritance (1). This is in accord with a complex set of interactions among the multiple pathways through which energy balance and glucose homeostasis are regulated. Insights into the pathophysiology of these disorders would therefore be facilitated by an understanding of the manner in which such pathways interact. For example, serotonin- and leptin-responsive pathways have both been implicated in the regulation of energy balance and glucose homeostasis, yet the extent to which they interact has been unclear.

Central serotonin [5-hydroxytryptamine (5-HT)] systems have long been implicated in the regulation of food intake and energy balance, as highlighted by the clinical use of the serotonin releaser and reuptake inhibitor fenfluramine as an appetite suppressant and weight loss medication (2). More recently a growing concern regarding the diabetogenic side effects of the atypical antipsychotics, which have nonspecific 5-HT receptor antagonist properties, has highlighted the need for an improved understanding of serotonin's influence on glucose homeostasis (3). A role for serotonin in glucose homeostasis is also consistent with prior studies indicating that fenfluramine improves glucose tolerance and insulin action (4).

The serotonin 2C receptor (5HT2CR), one of at least 14 distinct subtypes of 5-HT receptors, has been shown to play an important role in serotonergic effects on energy balance. Expression of 5HT2CR is restricted to the central nervous system,

in which it is found in both hypothalamic and extrahypothalamic regions implicated in energy balance and glucose regulation (5, 6). Mice with targeted null mutations of the *htr2c* gene (2C mutant mice) are resistant to the anorectic effects of fenfluramine (7). In addition, 2C mutant mice are hyperphagic and develop mild obesity, hyperinsulinemia, and impaired glucose tolerance by the age of 6 months (8). These deficits are accelerated and exaggerated in 2C mutant mice fed a high-fat diet, consistent with the possibility that their hyperinsulinemia and glucose intolerance results indirectly as a consequence of their elevated adiposity (8).

Several lines of evidence support the possibility that serotonin and leptin may regulate energy balance and glucose homeostasis by influencing common neural pathways. Both leptin receptors and 5HT2CRs are found within multiple hypothalamic structures implicated in energy balance. These include the ventromedial, dorsomedial, paraventricular, lateral, and arcuate hypothalamic nuclei (5, 6, 9, 10). Notably, leptin receptors expressed in the arcuate nucleus have been shown to play an important role in glucoregulation (11). 5HT2CRs are also expressed in this region, providing a potential site at which leptin and serotonin systems could interact to regulate glucose homeostasis.

To investigate potential interactions between 5HT2CR- and leptin-responsive pathways on the regulation of energy balance and glucose homeostasis, we crossed mice with null mutations of the *htr2c* gene with animals bearing null mutations of the leptin (*lep*) gene to generate mice deficient in both genes (OB2C double mutant mice). Phenotypic analysis of these animals and the effects of fenfluramine in wild-type and 2C mutant mice led us to report here that 5HT2CRs mediate central serotonergic influences on peripheral glucose homeostasis in a manner that is influenced by leptin signaling.

First Published Online November 26, 2007

Abbreviations: 5-HT, 5-Hydroxytryptamine; 5HT2CR, serotonin 2C receptor; wild-type experimental mice.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

Materials and Methods

Animals

Male mice heterozygous for the obese spontaneous mutation (*Lep^{ob}*) were obtained from the Jackson Laboratory (B6.V-*Lep^{ob}*/J; Bar Harbor, ME) and bred with female mice heterozygous for a null mutation of the X-linked *htr2c* gene (12). Both mutations were congenic on a C57BL/6J background. From this cross, female mice heterozygous for both the *Lep^{ob}* and *htr2c*[−] mutations (*htr2c*[−]/*htr2c*⁺, *Lep^{ob}*/*Lep*⁺) and male mice heterozygous for the *lep* mutation (*Lep^{ob}*/*Lep*⁺) were then bred to produce the male experimental mice (WT: *htr2c*⁺/*Y*, *Lep*⁺/*Lep*⁺; 2C: *htr2c*[−]/*Y*, *Lep*⁺/*Lep*⁺; OB: *htr2c*⁺/*Y*, *Lep^{ob}*/*Lep^{ob}*; OB2C: *htr2c*[−]/*Y*, *Lep^{ob}*/*Lep^{ob}*) and heterozygous mice for additional breeding. For pharmacological studies, experimental mice were generated by breeding female mice heterozygous for the *htr2c*[−] mutation with male C57BL6/J mice obtained from the Jackson Laboratory. Genotyping for the *htr2c*[−] mutation was performed by PCR analysis using a primer within the neomycin resistance gene (NeoD: 5′-CACCTTGCTCCTGCCGAGAAA-3′) and flanking primers within the *htr2c* gene (2C forward: 5′-GCTCA-GAATTCTGGAAATGTGT-3′; 2C reverse: 5′-CGGACTGCTAAATTGGGTC-3′) to produce a 114-bp band for WT mice and a 600-bp band for 2C mutant mice. Genotyping for the obese mutation was performed as previously described (13). All animals were housed at 20–24°C on a 12-h light, 12-h dark cycle (lights on at 0700 h) with free access to water and a standard chow diet (PicoLab Mouse Diet 20 5150; Purina Mills, Richmond, IN) except where indicated. Experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals and the University of California, San Francisco, Institutional Animal Care and Use Committee.

Food and water intake measurements

Mice were individually housed for 16 d in cages with feeders and water bottles mounted at one end. Animals were weighed before placement in the monitoring apparatuses and again at the end of data collection period. Intake of food and water were determined daily. Mean intake for the last 12 d of data collection was used for group comparisons to allow 4 d of acclimation to the housing conditions. The mice were run in 12 cohorts of three to 21 mice determined by the intermittent generation of mice from the breeding colony. The mice ranged in age from 2 to 8 months (2 months: WT, *n* = 10, 2C, *n* = 10, OB, *n* = 15, OB2C, *n* = 10; 3 months: WT, *n* = 4, 2C, *n* = 2, OB, *n* = 6, OB2C, *n* = 4; 4 months: WT, *n* = 7, 2C, *n* = 10, OB, *n* = 10, OB2C, *n* = 7; 5 months: WT, *n* = 3, 2C, *n* = 5, OB, *n* = 6, OB2C, *n* = 3; 6 months: WT, *n* = 3, 2C, *n* = 3, OB, *n* = 1, OB2C, *n* = 2; 7 months: WT, *n* = 2, 2C, *n* = 5, OB, *n* = 4; OB2C, *n* = 4; 8 months: WT, *n* = 4, 2C, *n* = 6, OB, *n* = 7; OB2C, *n* = 4), and where possible, individual mice were tested at multiple ages (one run: *n* = 47 mice; two runs: *n* = 29; three runs: *n* = 12; four runs: *n* = 4) to assess changes in intake with age.

Urine glucose

Mice were removed between 1300 and 1700 h from group housing in which food and water were available *ad libitum* and singly housed in a clean cage without bedding, food, or water. Urine produced was applied to a urinalysis reagent strip (Diastix; Bayer, Elkhart, IN) and scored on a color scale for glucose (0, 100, 250, 500, 1000, 2000 mg/dl). Mice were tested between 2 and 8 months of age (2 months: WT, *n* = 6, 2C, *n* = 14, OB, *n* = 13, OB2C, *n* = 11; 3 months: WT, *n* = 8, 2C, *n* = 9, OB, *n* = 6, OB2C, *n* = 7; 4 months: WT, *n* = 1, 2C, *n* = 6, OB, *n* = 6, OB2C, *n* = 7; 5 months: WT, *n* = 8, 2C, *n* = 8, OB, *n* = 12, OB2C, *n* = 10; 6 months: WT, *n* = 5, 2C, *n* = 8, OB, *n* = 8, OB2C, *n* = 6; 7 months: WT, *n* = 4, 2C, *n* = 1, OB, *n* = 5, OB2C, *n* = 4; 8 months: WT, *n* = 6, 2C, *n* = 4, OB, *n* = 2, OB2C, *n* = 8).

Fasting serum physiology

Mice were weighed and individually housed for 16 h before an 8-h fast beginning at 0700 h. Mice were then rapidly decapitated for collection of trunk blood. Samples were centrifuged to collect serum for measurement of glucose, insulin, glucagon, and corticosterone. Mice were tested from 2 to 10 months of age (2 months: WT, *n* = 7, 2C, *n* = 8, OB, *n* = 10, OB2C, *n* = 11; 4 months: WT, *n* = 6, 2C, *n* = 8, OB, *n* =

11, OB2C, *n* = 8; 6 months: WT, *n* = 6, 2C, *n* = 10, OB, *n* = 12, OB2C, *n* = 9; 10 months: WT, *n* = 11, 2C, *n* = 10, OB, *n* = 15, OB2C, *n* = 7). Glucose was measured using a GM7 Analox instrument (Analox Instruments Ltd., Lunenburg, MA) using the glucose oxidase method. Insulin was measured using the ALPCO ultrasensitive mouse insulin enzyme immunoassay no. 10-1150-01 (ALPCO Diagnostics, Salem, NH). Glucagon and corticosterone were measured by RIA using the Linco glucagon RIA kit GL-32K (Linco Research, St. Charles, MO), and a rat and mouse corticosterone RIA kit (no. 07-120103; MP Biomedicals, Solon, OH).

Weaning blood glucose

Mice were removed from their cages at 3 wk of age, and a drop of tail blood was applied to a One Touch Ultra glucometer (LifeScan Johnson & Johnson, Milpitas, CA) to measure blood glucose levels (WT: *n* = 8; 2C: *n* = 16; OB: *n* = 18; OB2C: *n* = 13).

Glucose tolerance tests

Beginning at 0700 h, mice were fasted for 8 h before blood collection from the tail vein for determination of baseline glucose levels (2 g/kg dose: WT, *n* = 15, 2C, *n* = 12, OB, *n* = 14, OB2C, *n* = 15; 0.2 g/kg dose: WT, *n* = 5, 2C, *n* = 5, OB, *n* = 7, OB2C, *n* = 10). Mice were then weighed and administered an ip injection of D-glucose (Sigma, St. Louis, MO) in distilled water. Subsequently blood was collected from the tail vein and centrifuged for serum collection at multiple time points for up to 2 h. For glucose tolerance tests with fenfluramine, 6 mg/kg fenfluramine or vehicle was administered ip immediately after baseline blood collection and before 2 g/kg glucose injection (WT, *n* = 8; 2C, *n* = 8). Fenfluramine, vehicle, and glucose were all injected at a volume of 10 μ l/g of body weight. Fenfluramine was obtained from Sigma and was dissolved in 0.9% NaCl. Serum glucose was measured using a Trinder glucose oxidase kit (Mega Diagnostics, Los Angeles, CA).

Insulin tolerance tests

Insulin tolerance tests were carried out only on OB and OB2C mice because the dose of insulin required to lower serum glucose in these mice would be lethal to nondiabetic mice. Beginning at 0700 h, mice were fasted for 8 h before blood collection from the tail vein for determination of baseline glucose levels (OB, *n* = 9; OB2C, *n* = 5). Mice were then weighed and administered an ip injection of 12 U/kg human insulin (Sigma) in distilled water. Subsequently blood was collected from the tail vein and centrifuged for serum collection at multiple time points for up to 2 h.

Pancreatic histology

Mice (OB, *n* = 5; OB2C, *n* = 6) were anesthetized with avertin (2–2–2-tribromoethanol) and perfused with 0.9% NaCl followed by 4% paraformaldehyde. Histological analysis, quantification of tissue area, and counting of cells were performed as described previously (14). Immunohistochemical and immunofluorescence analyses were performed on paraffin sections as described previously (15). The following primary antibodies were used: guinea pig antiinsulin diluted 1:500 (Linco Research), rabbit antiglucagon diluted 1:500 (Linco Research). The following secondary antibodies were used for immunofluorescence: fluorescein isothiocyanate-conjugated anti-guinea pig (Invitrogen, Carlsbad, CA); Cy3-conjugated antirabbit diluted 1:500 (Invitrogen). Glucagon- and insulin-positive cells were quantitated using OpenLab software (Improvision, Lexington, MA).

Glucose-stimulated insulin release

Eleven-week-old mice (OB, *n* = 5; OB2C, *n* = 7) were euthanized by cervical dislocation, and pancreases were immediately inflated by injection of 3 ml of collagenase into the bile duct. The pancreases were removed and digested in collagenase at 37°C for 13–17 min followed by straining, centrifugation, and washing. Islets were then isolated by Ficoll gradient centrifugation, and six medium-sized islets were picked into 5-ml, round-bottom tubes containing RPMI 1640 media with 0.1% BSA. Between three and six tubes of islets were used for each animal at each

concentration. Stock glucose was added to each tube to achieve concentrations of 75, 150, 300, and 600 mg/dl. Samples were then incubated with shaking for 1 h at 37°C followed by brief centrifugation and removal of supernatant for measurement of insulin by RIA using the Linco rat insulin RIA kit no. RI13K (Linco Research).

Statistics

Analyses of body weight, chow, and water intake were performed using a mixed-linear model (mixed procedure; SAS Institute Inc., Cary, NC) with mouse, *htr2c*, and *lep* genotypes, and age in months defined as class variables and age as a repeated measure. The analysis of urine glucose levels was also carried out using a mixed-linear model with mouse, *htr2c* and *lep* genotypes, age in months, and urine glucose defined as class variables and age as a repeated measure. ANOVA was used for comparing fasting serum values (SPSS, Chicago IL). The analyses of the glucose tolerance tests, insulin tolerance tests, and glucose-stimulated insulin release used repeated-measures ANOVA (SPSS). In the case of the glucose tolerance tests with fenfluramine, each mouse received vehicle and drug treatment in a crossover design, with a 1-wk interval between tests, and the difference between the glucose levels for drug treatment and vehicle treatment at each time point was analyzed by repeated-measures ANOVA (SPSS).

Results

Regulation of food and water intake

To determine how mutations in the *htr2c* and *lep* genes interact in the regulation of ingestion, daily food and water intake were measured in WT, 2C mutant, OB mutant, and OB2C double-mutant mice ranging in age from 2 to 8 months. These measurements revealed significant effects of age and the *htr2c*[−] and *Lep*^{ob} mutations on both food and water intake (Fig. 1, A and B). In addition, a significant synergistic interaction of the two mutations with age was observed for both food and water intake. OB2C double-mutant mice exhibited a large nonadditive increase in intake that declined slowly with age, compared with the WT and single-mutant mice (Fig. 1, A and B). This increase was greater than predicted based on the phenotypes of the single mutant lines and was particularly striking for water intake. For water intake, there was both a significant interaction of the two mutations with age and an interaction of the two genes across all ages. Thus, despite the decline in water intake to similar levels for all groups by 5 months of age, a significant interaction between the two genes across all ages is still detected [average water intake all mice from 2 to 8 months (mean ± SE): WT, 4.3 ± 0.1; 2C, 4.2 ± 0.1; OB, 4.9 ± 0.3; OB2C, 8.0 ± 0.8]. A similar synergistic interaction was not observed for body weight. Although significant effects of age and *htr2c* and *lep* genotype on body weight were observed, OB2C mutant mice were not heavier than expected based on the phenotypes of the single mutants alone (Fig. 1C).

Glucose homeostasis

Glucose levels were determined in the urine of WT, 2C, OB, and OB2C mice from 2 to 8 months of age. A significant effect of age and the *Lep*^{ob} mutation was observed on urine glucose levels. In addition, there was a significant interaction between the *htr2c*[−] and *Lep*^{ob} mutations, with the OB2C double mutants exhibiting elevated urine glucose levels not predicted by a simple additive effect of the single gene mutations (Fig. 2). This interaction indicates that the combined

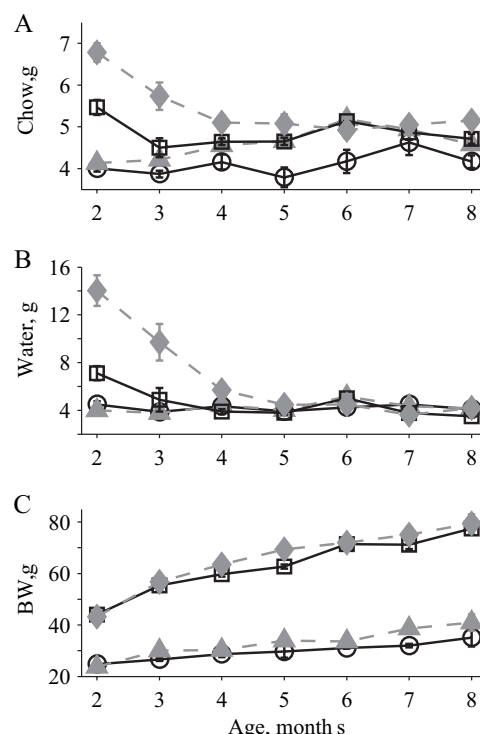


FIG. 1. Chow and water intake and body weight in WT (circles, solid line), 2C (triangles, dashed line), OB (squares, solid line), and OB2C (diamonds, dashed line) mice. A, Average daily chow intake. There was a significant effect of both *htr2c* and *lep* genotypes and a synergistic interaction of the two with age (*htr2c*, $P < 0.0001$; *lep*, $P < 0.0001$; age, $P = 0.0001$; *htr2c* × *lep*, $P = 0.8$; *htr2c* × age, $P = 0.5$; *lep* × age, $P < 0.0001$; *htr2c* × *lep* × age, $P = 0.004$). B, Average daily water intake. There was a significant effect of both *htr2c* and *lep* genotypes and a synergistic interaction of the two (*htr2c*, $P < 0.0001$; *lep*, $P < 0.0001$; age, $P < 0.0001$; *htr2c* × *lep*, $P < 0.0001$; *htr2c* × age, $P = 0.0006$; *lep* × age, $P < 0.0001$; *htr2c* × *lep* × age, $P < 0.0001$). C, Body weight (BW). There was a significant effect of both *htr2c* and *lep* genotypes without a significant interaction of the two (*htr2c*, $P = 0.007$; *lep*, $P < 0.0001$; age, $P < 0.0001$; *htr2c* × *lep*, $P = 0.8$; *htr2c* × age, $P = 0.1$; *lep* × age, $P < 0.0001$; *htr2c* × *lep* × age, $P = 0.8$).

effect of the two mutations produced a synergistic impairment in glucose homeostasis.

To confirm the presence of a synergistic impairment in the OB2C double mutants, fasting serum physiological parameters in WT, 2C, OB, and OB2C mice were investigated. There were significant effects of age and *htr2c* and *lep* genotype on fasting serum glucose levels. Again, a significant interaction between the two mutations on glucose levels was observed, with the OB2C mutants exhibiting a nonadditive increase in glucose levels relative to the WT and single-mutant mice (Fig. 3A).

The elevated fasting glucose levels of the OB and OB2C mutant mice declined with age at a similar rate, and by 10 months no differences in glucose levels were detected among the groups (Fig. 3A). The improvement in fasting glucose with age in OB mice is consistent with previous work, indicating that the diabetes phenotype of OB mice on a C57BL/6 background normalizes with age (16). To investigate when the diabetes phenotype in these mice develops, blood glucose levels were determined at weaning when mice were 3 wk old. At this age, there were no significant effects

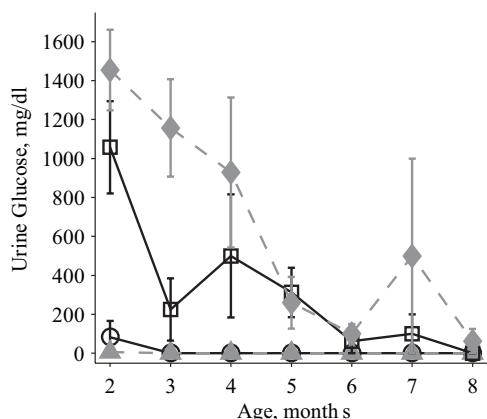


FIG. 2. Urine glucose in WT (circles, solid line), 2C (triangles, dashed line), OB (squares, solid line), and OB2C (diamonds, dashed line) mice. There was a significant effect of *lep* genotype and an interaction of *lep* and *htr2c* genotypes (*htr2c*, $P = 0.051$; *lep*, $P < 0.0001$; age, $P < 0.0001$; *htr2c* \times *lep*, $P = 0.04$; *htr2c* \times age, $P = 0.99$; *lep* \times age, $P < 0.0001$; *htr2c* \times *lep* \times age, $P = 0.99$).

of the *htr2c* or *lep* genotypes on blood glucose levels [blood glucose, milligrams per deciliter (mean \pm SE): WT, 151 ± 16 ; 2C, 169 ± 12 ; OB, 186 ± 11 ; OB2C, 182 ± 13 ; ANOVA: *htr2c*, $P = 0.6$, *lep*, $P = 0.07$, *htr2c* \times *lep*, $P = 0.4$]. This indicates that the diabetes phenotype develops after weaning, between the ages of 3 and 8 wk.

In contrast with the alterations in glucose levels, insulin levels were significantly altered only by the *Lep^{ob}* mutation (Fig. 3B). Glucagon and corticosterone were also measured at

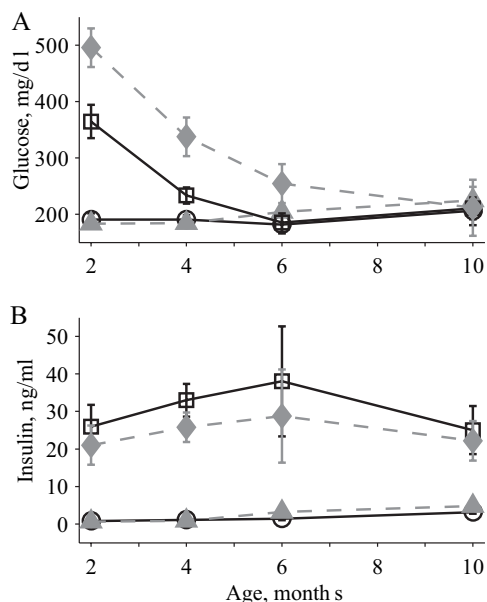


FIG. 3. Fasting serum glucose and insulin over time in WT (circles, solid line), 2C (triangles, dashed line), OB (squares, solid line), and OB2C (diamonds, dashed line) mice. A, Serum glucose. There was a significant effect of both *htr2c* and *lep* genotypes and a synergistic interaction of the two (*htr2c*, $P = 0.001$; *lep*, $P < 0.0001$; age, $P < 0.0001$; *htr2c* \times *lep*, $P = 0.007$; *htr2c* \times age, $P = 0.5$; *lep* \times age, $P < 0.0001$; *htr2c* \times *lep* \times age, $P = 0.1$). B, Serum insulin. There was a significant effect only of the *lep* genotype (*htr2c*, $P = 0.5$; *lep*, $P < 0.0001$; age, $P = 0.7$; *htr2c* \times *lep*, $P = 0.3$; *htr2c* \times age, $P = 0.99$; *lep* \times age, $P = 0.6$; *htr2c* \times *lep* \times age, $P = 0.99$).

2 months of age, when the difference in glucose levels between OB and OB2C mutants was largest. At this age, only the *Lep^{ob}* mutation resulted in significant elevations of insulin, glucagon, and corticosterone (Table 1). In contrast, there were significant effects of both *htr2c⁻* and *Lep^{ob}* mutations as well as a significant interaction between the mutations on glucose levels at this age.

A glucose tolerance test performed on WT, 2C, OB, and OB2C mice with 2 g/kg glucose also revealed a significant nonadditive effect of the combined mutations on glucose levels (Fig. 4A). Glucose levels in both the OB and OB2C mutant mice returned to baseline values more slowly in comparison with the WT and 2C mutant mice, as revealed by the significant interaction of the *Lep^{ob}* mutation with time. In contrast, a three-way interaction between time and the two gene mutations was not significant, indicating that the significant interaction of the two mutations was attributable to the altered baseline glucose levels (Fig. 4A). Similar effects were seen with a lower glucose dose of 0.2 g/kg (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online Web site at <http://endo.endojournals.org>). An insulin tolerance test performed on OB and OB2C mice showed significant effects of time and *htr2c⁻* mutation and a significant interaction of the two (Fig. 4B), indicating that OB2C mice had altered sensitivity to an insulin challenge, compared with OB mice.

Pancreatic islet morphology and function

To determine whether the OB2C double-mutant phenotype was associated with alterations of pancreatic morphology, pancreatic tissue from OB and OB2C double mutants was examined at 11 wk of age, when phenotypic differences in glucose levels were largest. No significant difference in islet number or total islet area was observed (number, mean \pm SE: OB, 18.6 ± 3 , OB2C, 15.6 ± 1.9 , $P = 0.5$; normalized area, mean \pm SE: OB, 1 ± 0.3 , OB2C, 0.6 ± 0.04 , $P = 0.2$), nor were there differences in the ratios of insulin-positive to glucagon-positive cells (insulin/glucagon, mean \pm SE: OB, 2.3 ± 0.4 , OB2C, 2.0 ± 0.4 , $P = 0.60$). To determine whether the islets of OB and OB2C mutant mice differed in their ability to release insulin, a glucose-stimulated insulin release study was also performed. Consistent with the lack of difference in pancreatic morphology, there was no significant difference in glucose-stimulated islet insulin production between the two groups (Fig. 5).

Glucose tolerance test with fenfluramine

The synergistic impairment of glucose homeostasis in OB2C mutants may be attributable to an indirect effect of increased adiposity or the result of a previously underappreciated role for 5HT2CRs in glucose homeostasis. However, no significant differences in the weights of adipose depots were observed between OB and OB2C mutants at 2 months of age (*t* tests, mean \pm SE, values in grams: epididymal OB, 3.8 ± 0.8 , OB2C, 3.9 ± 1.2 , $P = 0.8$; perirenal OB, 1.1 ± 0.5 , OB2C, 1.4 ± 0.3 , $P = 0.1$; omental OB, 1.1 ± 0.2 , OB2C, 1.2 ± 0.2 , $P = 0.2$; femoral OB, 1.5 ± 0.3 , OB2C, 1.7 ± 0.3 , $P = 0.2$) despite large differences in glucose levels. This suggests that 5HT2CRs may have a role in glucoregulation

TABLE 1. Fasting serum physiological parameters at 2 months of age

Measurement	WT	2C	OB	OB2C	2c, <i>P</i> value	<i>lep</i> <i>P</i> value	2c × <i>lep</i> , <i>P</i> value
Glucose, mg/dl	191 ± 31	183 ± 29	364 ± 26	496 ± 25	0.03	<0.0001	0.02
Insulin, ng/ml	0.7 ± 5	0.6 ± 5	26 ± 4	20 ± 4	0.6	<0.0001	0.6
Glucagon, pg/ml	35 ± 23	38 ± 16	136 ± 16	91 ± 13	0.3	<0.0001	0.2
Cort, ng/ml	77 ± 25	68 ± 24	248 ± 21	301 ± 20	0.3	<0.0001	0.2

Measurements reported are the mean and SEM. Significance values are from 1 × 2 ANOVA for each measurement.

that is independent of phenotypic differences in adiposity. To further evaluate this hypothesis, we examined the effect of fenfluramine on glucose tolerance in young WT and 2C mutant mice at an age before the development of phenotypic differences in adiposity. 2C mutant mice and their WT littermates were injected with either vehicle or 6 mg/kg fenfluramine and then administered a glucose tolerance test. Fenfluramine treatment produced a more rapid decline toward baseline glucose levels in WT mice, compared with 2C mutant mice, as revealed by the significant interaction of the *htr2c*[−] mutation with time (Fig. 6). This differential response of the WT and 2C mutant mice to fenfluramine occurred at an age when the mice were not diverged in body weight (*t* test, mean ± SE, values in grams: WT, 22.0 ± 0.7, 2C, 23.6 ± 0.6, *P* = 0.09), indicating that the 5HT2CR has a role in regulation of glucose homeostasis that is independent of its role in the regulation of food intake and body weight.

Discussion

We report that combined mutations of the *lep* and *htr2c* genes results in a synergistic impairment of glucose ho-

meostasis, accompanied by a synergistic increase in water intake in OB2C mice, indicating that leptin and the 5HT2CR interact in glucoregulation. The differences in glucose levels and food and water intake seen between OB and OB2C mice were not attributable to phenotypic differences in adiposity, suggesting a body weight-independent role of the 5HT2CR in regulation of glucose homeostasis. In this study, the elevation of body weight in 2C mutant mice, although significant, is somewhat less than reported earlier (8). Factors in the present study that may account for this include the exposure of animals to periods of individual housing and potential maternal effects of heterozygosity for the *Lep*^{ob} mutation in dams from which experimental animals were derived.

Several prior functional and neuroanatomical studies raised the possibility that interactions between leptin- and serotonin-responsive neuronal pathways could exist. OB mice were reported to have decreased expression of serotonin transporter mRNA in the dorsal raphe (17), and leptin treatment of OB mice increased the concentration of serotonin in hypothalamic and brain stem structures (18). In another study, a mouse line modeling leptin resistance (induced by constitutive leptin overexpression) was crossed with 2C mutant mice, resulting in enhanced sensitivity to high-fat diet-induced obesity and hyperinsulinemia (19). Notably, both the 5HT2CR and leptin receptors are expressed in hypothalamic regions implicated in energy balance and glucose homeostasis, including the arcuate nucleus of the hypothalamus, the dorsomedial hypothalamus, the paraventricular hypothalamus, and the lateral hypothalamus (5, 6, 9, 10). Coexpression of 5HT2CRs and leptin receptors has also been shown to occur at the cellular level: leptin receptors are expressed on serotonergic neurons in the raphe nuclei (15, 20,

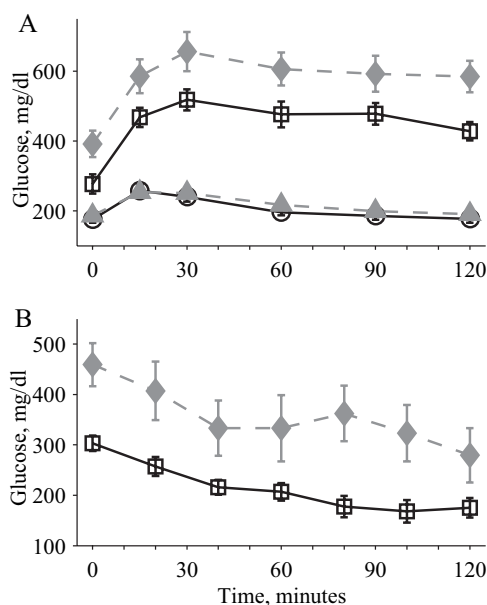


FIG. 4. A, Glucose tolerance test with 2 g/kg glucose in 2-month-old WT (circles, solid line), 2C (filled triangles, dashed line), OB (squares, solid line), and OB2C (filled diamonds, dashed line) mice. There was a significant effect of both *htr2c* and *lep* genotypes and a significant interaction between the two (*htr2c*, *P* = 0.008; *lep*, *P* < 0.0001; time, *P* < 0.0001; *htr2c* × *lep*, *P* = 0.03; *htr2c* × time, *P* = 0.7; *lep* × time, *P* < 0.0001; *htr2c* × *lep* × time, *P* = 0.9). B, Insulin tolerance test with 12 U/kg in 10-wk-old OB (squares, solid line) and OB2C (filled diamonds, dashed line) mice. There was a significant effect of *htr2c* (*P* = 0.008), time (*P* < 0.0001), and a significant interaction of *htr2c* × time (*P* = 0.03).

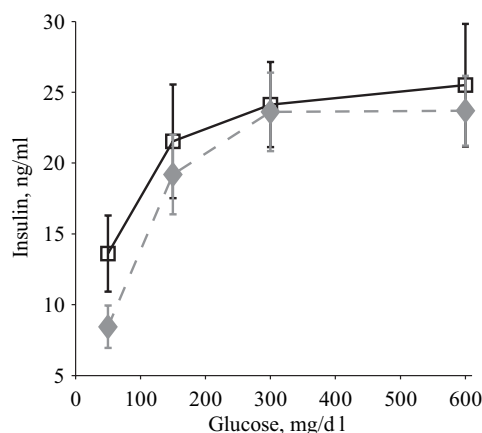


FIG. 5. Glucose-stimulated insulin release from isolated pancreatic islets of 2-month-old OB (squares, solid line) and OB2C (diamonds, dashed line) mice. There was a significant effect of glucose but not *htr2c* genotype (*htr2c*, *P* = 0.3; glucose, *P* < 0.0001; *htr2c* × glucose, *P* = 0.9).

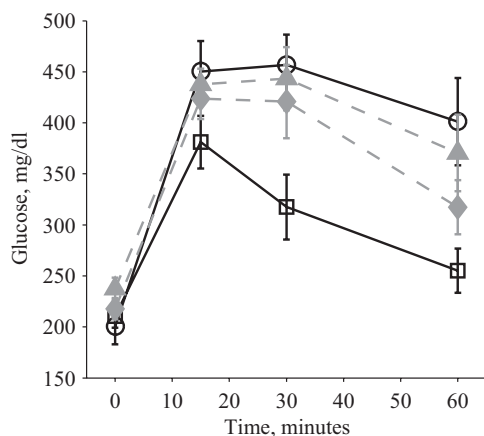


FIG. 6. Glucose tolerance test with 2 g/kg glucose with 6 mg/kg D-fenfluramine or vehicle in 3-month-old WT and 2C mice. Shown on graph are WT with vehicle (circles, solid line), WT with D-fenfluramine (squares, solid line), 2C with vehicle (triangles, dashed line), and 2C with D-fenfluramine (diamonds, dashed line). There was a significant interaction of *htr2c* genotype with time on the difference in glucose levels between saline and D-fenfluramine treatment (*htr2c*, $P = 0.1$; time, $P < 0.0001$; *htr2c* \times time, $P = 0.01$).

21), and these serotonergic neurons have been shown to accumulate leptin (22). 5HT_{2C}Rs are also expressed on leptin-sensitive proopiomelanocortin neurons in the arcuate nucleus (23), a brain region through which leptin regulates glucose homeostasis (11). The 5HT_{2C}CR does not appear to be expressed outside the central nervous system, and quantitative RT-PCR on mouse pancreas tissue did not reveal any expression of 5HT_{2C}CR mRNA, both in our hands (data not shown) and elsewhere (24).

At 2 months of age, OB2C double-mutant mice had dramatically increased food (124% of OB intake) and water (198% of OB intake) intake, compared with their OB littermates. In addition, OB2C double mutants exhibited elevated urine glucose levels as well as higher fasting glucose and impaired glucose tolerance, demonstrating a synergistic interaction of the *lep* and *htr2c* genes in the regulation of glucose homeostasis.

The impaired glucose homeostasis phenotype of both OB and OB2C mice ameliorates as the mice age. This finding had previously been observed in OB mice on the C57BL/6 background (16); however, the mechanisms underlying this age-dependent effect have not been fully delineated. Interestingly, serum insulin concentrations do not appear to change significantly with age (Fig. 3B), raising the possibility that euglycemia seen in older OB mice may result from improved insulin sensitivity, rather than increased insulin production. In parallel with the improvement of glucose levels with age, both OB and OB2C mice exhibit a normalization of food and water intake. Given the well-established effects of elevated blood glucose on osmotic regulation, it is likely that the normalization of water intake is secondary to normalization of serum glucose levels. Because hyperphagia is also linked to diabetes mellitus (25–27), the normalization of feeding in these mice may also occur secondary to their improvement in glucose homeostasis. Despite the marked hyperphagia of young adult OB2C mice, their body weights did not differ from those of OB mice. This is consistent with the notion that

attempts to compensate for energetic losses from urinary glucose excretion contribute to hyperphagia in mice of both genotypes (25).

To better examine the synergistic interaction between the *lep* and *htr2c* genes, we focused attention on 2-month-old mice, the age at which their diabetes is maximal. We did not see any differences between 2-month-old OB and OB2C mice in pancreas morphology or the ability of islets to respond *in vitro* to glucose (Fig. 5), suggesting that the double mutant phenotype is unlikely attributable to a defect in pancreatic development or intrinsic islet cell function. However, this does not exclude the possibility that central regulation of pancreatic function may differ in OB and OB2C mice, *i.e.* through effects of central serotonin on the sympathetic and parasympathetic nervous systems (28).

It is also possible that phenotypic influences on insulin sensitivity contribute to the exacerbation of diabetes in OB2C mice. Consistent with this possibility, an insulin tolerance test indicated altered sensitivity to injected insulin in OB2C mice, compared with OB mice. However, interpretation of these insulin tolerance test results is confounded by the significantly elevated (relative to OB) fasting glucose levels of OB2C mice. It is noteworthy, however, that despite the higher fasting serum glucose levels of the double mutants, the fasting serum insulin levels of two groups do not differ. This is consistent with the possibility that reduced insulin sensitivity contributes to the enhanced disruption of glucose homeostasis in OB2C mice.

Whereas a null mutation of the *htr2c* gene does not result in impairment of glucose homeostasis in young, lean mice, Nonogaki *et al.* (8) showed that obese 2C mutant mice do develop impaired glucose homeostasis. However, it was unclear whether this reflected a direct impairment by the *htr2c*[−] mutation or an indirect consequence of enhanced adiposity, which is in and of itself a major risk factor for type 2 diabetes (29). Our observation that the effects of fenfluramine on glucose tolerance are blunted in 2C mutant mice indicates that manipulation of the 5HT_{2C}CR can play a role in glucose regulation in the absence of obesity. This suggests that young, lean 2C mutant mice, which have normal glucose tolerance, are somehow able to compensate for their chronic lack of 5HT_{2C}CR. This compensation appears to fail in diet-induced obesity [a condition accompanied by leptin resistance (8)], in a genetic model of leptin resistance (19), and in the absence of leptin (this study). These data suggest that the manner in which 2C mutant mice maintain glucose homeostasis is leptin dependent and that impairment of the leptin system augments the impact of 5HT_{2C}CR signaling on glucose homeostasis.

In summary, our findings indicate that serotonin and leptin interact synergistically to regulate glucose homeostasis. These findings may have significant clinical implications because diabetes and obesity associated with atypical antipsychotic drug treatment pose substantial obstacles in the treatment of schizophrenia and bipolar disorder (4). Atypical antipsychotic drugs are high-affinity antagonists of the 5HT_{2C}CR (30), and 5HT_{2C}CR occupancy has been found to correlate with the obesigenic and diabetogenic effects of these drugs (31). Furthermore, our finding that the 5HT_{2C}CR interacts with the leptin system to regulate glucose ho-

meostasis further establishes the serotonin system as a possible target for the treatment of diabetes mellitus itself.

Acknowledgments

We thank the University of California, San Francisco Diabetes and Endocrine Research Islet Core for mouse pancreatic islet isolation; Janet Lau for assistance with pancreatic histology; and Elaine Storm, Elinor Sullivan, and Luna Abdallah for critical reading of the manuscript.

Received July 9, 2007. Accepted November 13, 2007.

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This work was supported by a National Alliance for Research on Schizophrenia and Depression Artworks Young Investigator Award (to E.H.G.) and an National Institutes of Health RO1 grant (to L.H.T.).

Disclosure Statement: The authors have nothing to disclose.

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